

Lipid Peroxidation Products and Antioxidants in Human Disease

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Lipid peroxidation (LPO) is a free radical-related process that in biologic systems may occur under enzymatic control, e.g., for the generation of lipid-derived inflammatory mediators, or nonenzymatically. This latter form is associated mostly with cellular damage as a result of oxidative stress, which also involves cellular antioxidants in this process. This article focuses on the relevance of two LPO products, malondialdehyde (MDA) and 4-hydroxynonenal (HNE), to the pathophysiology of human disease. The former has been studied in human serum samples of hepatitis C virus-infected adults and human immunodeficiency virus-infected children. In these two cases it is shown that the specific assay of serum MDA is useful for the clinical management of these patients. The presence of MDA in subretinal fluid of patients with retinal detachment suggests the involvement of oxidative stress in this process. Moreover, we were able to report the dependence of this involvement on the degree of myopia in these patients. The assay of MDA contents in the peripheral nerves of rats fed a chronic alcohol-containing diet or diabetic mice also confirms the pathophysiologic role of oxidative stress in these experimental models. In these two cases, associated with an increase in tissue LPO products content, we detected a decrease of glutathione peroxidase (GSHPx) activity in peripheral nerve, among other modifications. We have demonstrated that *in vitro* HNE is able to inhibit GSHPx activity in an apparent competitive manner, and that glutathione may partially protect and/or prevent this inactivation. The accumulation of LPO products in the brain of patients with Alzheimer's disease has also been described, and it is on the basis of this observation that we have tried to elucidate the role of oxidative stress and cellular antioxidants in β -amyloid-induced apoptotic cell death of rat embryo neurons. Finally, we discuss the possible role of the observed vascular effects of HNE on human arteries. — *Environ Health Perspect* 106(Suppl 5):1229–1234 (1998). <http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-5/1229-1234romero/abstract.html>

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Introduction

Inflammation associated with infectious and degenerative diseases, and probably others, leads to the activation of the so-called inflammatory cells. This activation by means of several mechanisms involving neutrophils, endothelial cells, etc., might

promote oxidative stress. Oxidative stress was originally defined as the disequilibrium between prooxidants and antioxidants in biological systems (1). Once this imbalance appears, cellular macromolecules may be damaged by the predominant free radicals.

This leads to oxidative modifications of the genome, proteins, structural carbohydrates, and lipids; in the latter case, lipid peroxidation (LPO) occurs. LPO is a free radical-related process, that in biologic systems may occur under enzymatic control, e.g., for the generation of lipid-derived inflammatory mediators, or nonenzymatically. This latter form is, as mentioned above, associated mostly with cellular damage as a result of oxidative stress, and a great variety of aldehydes is formed when lipid hydroperoxides break down in biological systems, among them, malondialdehyde (MDA) and 4-hydroxynonenal (HNE) (2).

To demonstrate involvement of oxidative stress as pathophysiologic mechanisms in diseases or experimental models, several approaches are possible. Because oxidative stress may lead to cellular damage, any marker of cellular disruption may confirm this role, although it may still be necessary to demonstrate directly the intervention of oxidative stress. This may be achieved by the direct detection of activated species *in situ*, the assay of end products of protein or lipid oxidation, or any other oxidative modification of macromolecules (modified bases of nucleotides, etc.). Further evidence for the role of oxidative stress may be obtained by studying the content and activity of antioxidants. In this review, our data attempt to demonstrate the crucial role of oxidative stress in different disease and experimental models. The goal is different for each model. In some, the detection of end products of LPO may help the clinical management of these patients; in others, oxidative stress participation is reported for the first time. Finally other results may help to understand pathophysiologic mechanisms that are observed in these diseases or experimental models.

We have studied serum MDA concentrations in two relevant viral infections that have in common the involvement of T cells: hepatitis C virus (HCV) in adults (3), and pediatric human immunodeficiency virus (HIV) infections (4). Moreover, we have demonstrated the involvement of oxidative stress in retinal detachment by detecting LPO products in subretinal fluid of patients undergoing surgery (5).

We have also demonstrated the pathophysiologic role of oxidative stress in experimental diabetic neuropathy (6) and

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Abbreviations used: AD, Alzheimer's disease; ALT, alanine aminotransferase; CDC, Centers for Disease Control and Prevention; CHC, chronic hepatitis C; GSH, glutathione; GSHPx, glutathione peroxidase; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HNE, 4-hydroxynonenal; IF, interferon $\alpha 2$ b; LPO, lipid peroxidation; MDA, malondialdehyde; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances.

chronic alcohol intoxication-induced polyneuropathy (7). Interestingly, in these two models LPO products accumulate in peripheral nerve and both show a decrease of glutathione peroxidase (GSHPx) activity in this same tissue. This finding led us to investigate the effect of HNE on this enzymatic activity in view of some recent reports showing the ability of this compound to inactivate other enzymes (8–11). Because LPO products accumulate in the brain of patients with Alzheimer's disease (AD), we studied the role of other cellular antioxidants in *in vitro* models for the toxicity of β -amyloid (the peptide accumulating in the brain of AD patients) (12,13), and in an experimental model of spontaneous apoptosis (14). Finally, we discuss the possible pathophysiologic role of the reported effects of HNE on two distinct vascular beds—human cerebral (15) and mesenteric (16) arteries.

Serum Malondialdehyde in Patients with Chronic Hepatitis C

It has been reported that serum LPO products are increased in patients with liver disease also in chronic hepatitis C (CHC) patients (20).

The existence of an agent causing non-A, non-B hepatitis was documented by Alter et al. (21) and named hepatitis C virus (HCV). Hepatitis C has some striking clinical features including variability of the serum activity of alanine aminotransferase (ALT). In most cases this variability does not correlate with the histopathologic findings of the hepatic biopsies of these patients (22). In addition, more than 50% of the patients with acute hepatitis C will develop chronic infection (23). Studies are limited on HCV infection markers in healthy populations, as most use healthy blood donors, but one could assume a variable prevalence ranging from 0.26 to 2.3% for anti-HCV in different parts of the world (24–26). Thus, HCV infection is a health problem worldwide. Moreover, some of these liver diseases progress eventually to cirrhosis or even degenerates into hepatocellular carcinoma (27,28). There have been many attempts to find serologic or cellular markers that may help in the clinical management and evaluation of treatment response of these patients, including plasma viral load (29,30), plasma anti-HCV IgM (31) or other soluble immune factors (32), hepatic viral RNA (33), etc. However, repeated biopsies are needed to evaluate progression of the disease (34).

We reported that serum LPO products were significantly increased in a selected group of patients with CHC before interferon (IF) α -2 treatment (20). LPO products, measured as thiobarbituric acid (TBA) reactive substances (TBARS) (20), and also MDA (3) concentrations decreased after IF- α 2 treatment (3000 U, 3 times a week for 6 months), as well as the ALT activity, in serum of these patients. When applying a specific assay of MDA (fluorescence detection of the MDA-TBA after chromatographic separation) (3,4), the differences in serum MDA before and after IF- α 2 treatment, in the responding and nonresponding IF- α 2 treatment groups of patients revealed that higher MDA values before IF- α 2 treatment have a higher probability for a better outcome than those with relatively lower values, as confirmed histopathologically (3).

These results confirm involvement of oxidative stress as part of the pathophysiology of CHC. The increase in serum MDA concentration in CHC patients may fit in well with the recently reported GSH depletion observed in hepatic tissue, plasma, and peripheral blood mononuclear cells of CHC patients (35). MDA values apparently correspond with the severity of the inflammatory findings whereas these histopathologic signs do not correlate well with the serum ALT activity (3). The fact may be speculated as the predominant contribution of the inflammatory cells over the hepatocytes to the overall lipid peroxidation rate. This speculation is reinforced by the serum ALT activity levels (more directly attributable to the hepatic cell damage) that rapidly normalize during IF- α 2 treatment. Serum MDA levels, which are significantly lower after IF- α 2 treatment, do not reach control values during or after treatment (3). The possible role of T cells in the development of chronicity in HCV-infected patients (23), acting probably as HCV reservoirs, has been recently confirmed (36), and again it fits with the above-mentioned lymphocytic glutathione depletion observed in CHC patients (35). Our results (3) should also be considered for the clinical management of CHC patients, as the effectiveness of IF- α 2 treatment could be additionally tested by serial-specific MDA determinations during the course of treatment.

Serum Malondialdehyde in Pediatric HIV Infection

Factors that correlate with the outcome of the human immunodeficiency virus

(HIV) infection are needed for a better understanding of the pathogenesis of this infection and certainly for a better clinical management of the infected individuals. This is of special relevance in particularly important for HIV seropositive children. HIV infection is associated with oxidative stress, in view, among other things, of the reduction of glutathione GSH contents in plasma and lung epithelial lining fluid (37) and T lymphocytes (38) of adult asymptomatic HIV seropositive individuals among other things. This decrease in GSH is also associated with an increase in the concentration of lipid peroxidation products in serum of these patients (39). Oxidative stress can occur early after the HIV infection (40). Moreover, oxidative stress might stimulate viral replication via nuclear factor kappa B in T lymphocytes (41), thus favoring the progression of the disease. It has also been demonstrated that some antioxidant substances might inhibit this activation sequence *in vitro*. These substances have been proposed as adjuvant therapy in HIV infection (42,43).

The HIV infection in children has some special features. Infants of HIV seropositive mothers are almost always seropositive at birth but might during the first 18 months of life turn seronegative if they are not infected (44). Early treatment of these children could improve life span. We have recently demonstrated (4) that HIV seropositive children have a higher serum malondialdehyde concentration than healthy children. In this preliminary study we further demonstrated that total antioxidant capacity of serum was decreased in HIV seropositive children but not always as significantly as serum MDA levels increased, compared with the control group. We have also developed a numerical model to classify these children according to the Centers for Disease Control and Prevention (CDC) criteria in which we assume the same distance between all clinical or immunologic disease stages (N, A, B, and C). We ascribed a numerical value to the CDC classification criteria (N = 1, A = 2, B = 3, and C = 4), thus transforming this variable into a continuous one. Under these conditions serum MDA levels correlated with the different clinical categories in each case. Interestingly, a high correlation was observed between these two criteria in our pediatric population (Spearman correlation coefficient $r = 0.953$) (45). We also showed that an apparent negative correlation ($r = -0.515$) could be established between MDA values and CD4⁺ lymphocyte total

count (46). These results are in agreement with what is expected compared to the published results for adults, i.e., that CD4⁺ T cells were the best single predictor of the progression to AIDS in HIV-infected adults (47,48). These results provide the basis for future studies that may prove this hypothesis and may help in the clinical management of these children.

LPO Products in Tissues as Oxidative Stress "Fingerprints"

LPO products and proteins accumulate in the subretinal fluid of patients undergoing retinal detachment surgery (5). The presence of MDA, and possibly other LPO products, in human subretinal fluid raises the questions of where do these substances come from and how does oxidative stress intervene in the pathophysiology of retinal detachment. It is generally accepted that retinal detachment is not normally associated with inflammation (49), a process where free radicals and lipid-derived intermediates play an important role (50,51). However, Hackett et al. (52) have shown that subretinal fluid stimulates *in vitro* migration and proliferation of retinal pigment epithelial cells, and Goto et al. (53) found that retinal structural membrane peroxidized lipids have chemotactic properties. Moreover, the presence of LPO products in the vicinity of the retina, e.g., in the subretinal fluid, might have vascular consequences (discussed below). This has been preliminarily demonstrated by the intravitreal injection into the rabbit eye of relatively low concentrations of HNE, showing an intense inflammatory response with impairment of the blood-retina barrier (54,55). The cellular elements most likely responsible for the initial generation of the necessary free radicals to induce LPO, or for the initial reaction to the presence of already generated free radicals, are retinal pigment epithelial cells, phagocytic cells that generate superoxide anion during phagocytosis of rod outer segments (56). The presence in the human retina of a relevant GSH content and GSH-associated conjugating activity towards HNE (57) may certainly play a role in the ultimate defense against oxidative damage and LPO, one of its direct consequences. The fact that hyaluronic acid depolymerization, associated with vitreous liquefaction, appears during rhegmatogenous retinal detachment (49), and that this depolymerization is induced by oxygen free radicals (58,59), again fits in the previous hypothesis.

This same approach, i.e., the evaluation of tissue LPO products levels in tissues, has been also applied to experimental models with similar results. We have focused in the last few years on the role of oxidative stress in peripheral nervous tissue affections, i.e., neuropathies. This has been reviewed elsewhere (60). Relevant to the present monograph, however, is the fact that associated with the accumulation of LPO products in peripheral nervous tissue in models of experimental diabetes (61) or chronic alcoholic intoxication (7), a decrease in GSHPx activity occurs in this same tissue (6,7,62,63), among other cellular antioxidants (64,65).

4-Hydroxynonenal Inhibits GSHPx

The alterations observed in GSHPx activity might be related to different mechanisms. However, the reported ability of HNE to modify proteins and therefore influence enzymatic activity, led us to study the possibility of a direct interaction between HNE and GSHPx. HNE reacts with GSH spontaneously, but within cells this reaction proceeds at a much higher rate, catalyzed by specific isoforms of GSH S-transferases (66,67). Moreover, reaction of this aldehyde with other amino acid residues leads to the modification of proteins and eventually to the inactivation of their enzymatic activity. It has been demonstrated the modification of histidine residues in insulin (8) after incubation with HNE (8), as well as the inactivation of glucose 6-phosphate dehydrogenase by the HNE-induced selective modification of an active-site lysine residue (9), the inhibition of Na,K-ATPase most probably by the interaction of the HNE with sulfhydryl groups (11), etc.

Incubation of GSHPx with HNE resulted in a loss of enzymatic activity, in a time- and concentration-dependent fashion. Over 90% of the inhibition observed for each of the different concentrations was achieved in the first 30 min, and no further loss of activity could be detected. We calculated the concentration that exerted 50% inactivation of the enzyme (I_{50}) as 0.12 mM. We tested whether the substrates would be able to protect the enzyme, and found that 1 mM GSH added 30 min prior to the addition of the aldehydes prevented GSHPx from inactivation almost completely. Furthermore, GSH reverted, the initial inactivation, at least partially, even when GSH was added after incubation with HNE. From the results of kinetic studies, it can be deduced that HNE is apparently able to competitively inhibit

GSHPx (68). The I_{50} value for HNE inhibition is in the range of those previously reported for several enzymes (69), and interestingly, coincides with the I_{50} value obtained for Na,K-ATPase (11). These authors proposed the contribution of cysteine residues in the inhibition of Na,K-ATPase according to the protection exerted by thiol reagents. GSHPx inhibition seems to be competitive. This is apparently in contrast with the results of the HNE-induced glucose 6-phosphate dehydrogenase inactivation, where a double reciprocal plot evidenced a noncompetitive mode of action for the inhibition exerted by glucose 6-phosphate on HNE-induced inactivation (9). In considering the data on GSH protection and the kinetic studies referred to above, it seems also more than reasonable that one or more amino acid residues not far from the active site might be modified. The most plausible candidate could be Lys or His, according to the proposed reactivity of these residues with HNE (8,9).

Pathophysiologic Relationship of Lipid Peroxidation Products and Antioxidants

Oxygen radicals and lipid peroxidation play a pivotal role in the observed damage during central nervous system trauma and stroke (70) (71), and more generally in neurotoxicity (72). Both oxygen radicals and peroxides are able to inactivate antioxidant enzymes (73). The role of GSH and GSH-related enzymes in the defense of neurons against oxidative burden has been repeatedly suggested (60,74,75). It has been recently reported that HNE has a role in β -amyloid-induced neuronal death (76), whereas it is also known that β -amyloid-induced apoptosis is associated with GSH depletion in primary neurons (12,13). The hypothesis of the involvement of oxidative stress in AD (77) is confirmed also by the finding of increased lipid peroxidation products levels in brains of these patients (78).

As mentioned above, HNE is able to impair Na,K-ATPase activity (11), and it has been proposed that HNE would mediate β -amyloid-induced oxidative damage to neuronal membrane proteins (76). As it has been recently demonstrated that β -amyloid-induced apoptosis is associated with GSH depletion in neurons (12,13), collectively, all these data demonstrate the pathophysiologic relevance of these findings. An additional HNE-induced inhibition of GSHPx in neurons that are already

challenged by β -amyloid may certainly close the vicious circle leading to cell death. Furthermore, it provides a rationale for considering selenoorganic compounds with GSHPx-like activity, such as ebselen (79,80), for the treatment of AD and probably other neurodegenerative diseases. Another open field is related to the mechanisms involved in this cell death process and its different forms, as antioxidants certainly are involved in cell death of different cell types in certain diseases mentioned in this monograph (HIV infection, AD, etc). The involvement of oxygen free radicals and cellular antioxidants such as GSH in the apoptotic process seems to be accepted by the majority of investigators. However, the sequence of intervention of these elements in the different models proposed up to now is still an open question. In this sense, we have recently reported the variations of cellular GSH levels in a model of spontaneous apoptosis—V79 multicellular spheroids (14).

Vascular Effects of Hydroxynonenal

LPO products such as lipid hydroperoxides (81) and aldehydes (15,82) show vascular effects in a variety of territories. Unlike reactive free radicals, aldehydes are rather

long-lived and can therefore spread from their site of origin and attack targets distant from the initial radical event (2) by circulating in blood. The action of HNE in endothelial cells has been studied in culture in terms of its cytotoxicity (83) and, in human cerebral arteries, from a functional point of view (15). The reported physiologic human plasma levels of HNE range from 0.3 to 0.7 μ M (84,85), and it has been demonstrated that levels increase in experimental alcoholic liver disease (86). An increase in circulating LPO products (determined as the TBARS), as it is observed in different human hepatic diseases (18) including hepatitis C (3,20) as mentioned above, would conceivably include a specific increase in circulating 4-HNE. We recently reported the relaxing effect of HNE on human mesenteric arterial segments, and its possible implications in the hemodynamic alterations observed during hepatic cirrhosis (16). These results confirm that the HNE relaxing effect is mostly mediated by the endothelium, as was previously reported in human cerebral arteries (15). HNE concentration in human biologic fluids has not been systematically studied in the disease state due to the difficulty of the procedures

described up to now (2,87). However, some evidence confirms that this aldehyde is increased in situations where other aldehydic LPO products are increased (88), and vice versa (89). The most frequent causes of portal hypertension and ascitis are liver diseases such as alcoholic hepatitis, cirrhosis, and cancer, etc. LPO is enhanced during all phases of human hepatic alcoholic disease, as can be inferred by the serum MDA increase during viral hepatitis (19,20), experimental hepatic alcoholic disease (86), etc. If the increase of LPO products concentration in serum supposes also an increase in HNE, the results mentioned above (16) would at least partially explain the hemodynamic alterations observed during portal hypertension. The increase in hydrostatic pressure does not completely explain the intense splanchnic arteriolar vasodilation responsible for the increase in cardiac output, arterial hypotension, hypervolemia, and decreased peripheral resistance found in these patients. The results presented support the proposal of HNE as one of the possible metabolites that contribute to these alterations. Further systematic studies are being undertaken to prove this hypothesis.

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